



An integrated strategy for polyploidization of *Cerastigma willmottianum* Stapf based on tissue culture and chemical mutagenesis and the carbon dioxide fixation ability of tetraploids

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Abstract

Cerastigma willmottianum Stapf (Plumbaginaceae, *Cerastigma*), a native Chinese plant, has decorative and medicinal values and is used in green areas and landscapes. However, wild plants have small flowers and scattered, pendulous branches with lower compactness, making them inferior as ornamentals. Polyploidization can improve ornamental properties and carbon sequestration capacity, providing novel germplasms with higher carbon dioxide assimilation abilities based on plant-based C biosequence. This study induced polyploidy in apical buds in vitro through tissue culturing and application of colchicine, trifluralin, or pendimethalin; in the most efficient protocol, explants were cultured for 7 days in MS medium supplemented with 1200.0 μM trifluralin and 2% dimethyl sulfoxide (DMSO), with a survival rate of 74.45% and polyploidization rate of 17.78%. After 5–8 successive generations obtained by cutting aseptic apical buds for purification, ploidy level was effectively and precisely identified by morphological observation, stomatal measurement, DNA content analysis, and chromosome counting. According to our breeding goal, the morphology and photosynthetic capacity of tetraploids were screened. Compared to diploids, tetraploids have improved ornamental properties, including greater compactness, larger flowers and heart-shaped leaves, and a greater net photosynthetic rate, with higher CO_2 assimilation efficiency. We developed an integrated protocol for the induction, purification, screening and identification of tetraploid *C. willmottianum* plants. Moreover, the newly developed tetraploid germplasm can be utilized to breed new cultivars. We hope that our research will lead to novel garden plants with improved ornamental value and high carbon sequestration ability, contributing to carbon neutrality.

Key message

An optimal integrated protocol for the polyploidization, purification, screening and identification of *C. willmottianum* was developed. A new tetraploid germplasm with improved ornamental value and high carbon sequestration ability was obtained.

Keywords *Cerastigma willmottianum* Stapf · Tissue culturing and chemical mutagenesis · Polyploidization · CO_2 assimilation efficiency · Ornamental value

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Abbreviations

CO ₂	Carbon dioxide
DMSO	Dimethyl sulfoxide
FCM	Flow cytometry
LAI	Leaf area index
PS I	Photosystem I
PS II	Photosystem II
Pn	Net photosynthetic rate
Ci	Intercellular carbon dioxide concentration
Gs	Stomatal conductance
Tr	Transpiration
WUE	Water use efficiency
VPD	Vapor pressure deficit
Fm	Maximum fluorescence
Fo	Minimal value of chlorophyll a fluorescence
Fv	Variable fluorescence of PS II
Fv/Fm	Maximal quantum yield of PS II
Fv/Fo	Maximum primary yield of photochemistry of PS II
Fm/Fo	The transportation of electrons of PS II

Introduction

After the first Industrial Revolution, the emission of CO₂, a representative greenhouse gas, has caused increasingly severe global warming. Therefore, countries around the world have proposed a concept called carbon neutrality, which aims to neutralize CO₂ emissions by afforestation and reductions in air pollution emission (Ravilious 2020). Plants absorb CO₂ and release O₂ through the process of photosynthesis. Green vegetation plays a critical role in offsetting atmospheric CO₂. Many flowering plants have multifunctional ecological and pharmaceutical values. As an increasingly environmentally friendly industry, the use of flowering plants has formed an intricate industrial chain, linking environmental protection, medical treatment and health care, which are predominant in the construction of human settlements and wellness (Kalluri et al. 2020; Niazi and Nalouisi 2020; Tuna et al. 2020; Zhang et al. 2020a). China, known as the ‘Mother of Gardens’, has bred unique and valuable varieties of wild plant resources, including *Cerastigma willmottianum* Stapf (Huang 2011; Li et al. 2014).

C. willmottianum Stapf (family: Plumbaginaceae; genus: *Cerastigma*), commonly known as purple lotus, is a perennial deciduous subshrub that is a native wild plant in China. It was first found in the Minjiang River Valley in Sichuan Province. Due to its strong resistance, extensive management, elegant purple flowers and long flowering period (from May to December), it is regarded as a valuable plant among endemic wild plants applied in landscaping. However, the branches of mature diploids (2n = 2x = 12) tend to be scattered and pendulous with lower compactness, which

decreases the ornamental value of the plant. The roots of *C. willmottianum* are rich in some chemically active ingredients, such as plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone), which has anticancer, antibacterial and other properties. Therefore, this species has been applied in folk medicine in China, India and other countries. Moreover, it is considered a Bach flower remedy plant and is made into a ‘Natural Therapy’ healthcare product that can soothe negative emotions (Li et al. 2014; Li 2016). Taken together, these results indicate that *C. willmottianum* is a multifunctional plant with ornamental, health care and medicinal values. However, to date, researchers have focused on its medicinal ingredients, tissue cultures and biological characteristics (Yue et al. 1997; Li 2016; Hu et al. 2019; Gao et al. 2021). Owing to the lack of germplasm innovation and research and the development of new cultivars, the promotion and application of *C. willmottianum* germplasm resources are limited.

The artificial induction of polyploidy is an effective and feasible breeding method to modify plant traits. It can cause changes in morphology (e.g., the ‘giga effect’, which causes gigantism of organs), physiological structure (stomatal size), biochemical substances in vivo (the contents of chlorophyll and photosynthetic active enzymes) and photosynthetic capacity. It has been reported that after polyploidization, the general capacity for CO₂ assimilation improves, and the photosynthetic capacity of polyploid plants is higher than that of the original diploid plants (Warner and Edwards 1989; Vyas et al. 2007; Iannicelli et al. 2020; Martin et al. 2019; Niazi and Nalouisi 2020). Therefore, polyploidization is a useful strategy for absorbing excess CO₂ from the atmosphere through the photosynthetic process to achieve carbon neutrality.

Induction, purification and identification are all indispensable parts of polyploid breeding. For induction, it is important to determine the best induction system, explant type and size and mutagen concentration and exposure duration (Eng and Ho 2019). To date, aside from the longest and most widely used chemical colchicine, herbicides such as pendimethalin and trifluralin have been increasingly used due to their small required doses and high effectiveness (Dhooghe et al. 2011; Eng and Ho 2019; Jiang et al. 2019). DMSO is a common penetrating agent and mutagen adjuvant. It can promote rapid cell penetrations by mutagens, significantly increasing the polyploidization rate and decreasing the chimerism rate (Dhooghe et al. 2011; Eng and Ho 2019). Moreover, to accelerate the process of polyploid breeding, it is important to understand how to purify induced seedlings and quickly identify their ploidy levels after induction. Currently, direct adventitious shoot regeneration and serial generation by cutting organs are commonly used methods for purification. Finally, polyploids are identified by stomatal measurement, flow cytometry (FCM) and chromosome

counting (Dhooghe et al. 2011; Eng and Ho 2019; Iannicelli et al. 2020; Niazi and Nalouisi 2020).

Based on the ability of polyploidization to modify plant traits and enrich the diversity of germplasm resources, this study induced polyploidy in *C. willmottianum* in vitro with a combination of tissue cultures and chemical mutagens (pendimethalin, trifluralin and colchicine). The objectives are threefold: (1) to develop a target-oriented protocol for polyploidization with efficient induction, fast purification and precise identification by comparing the survival rate and tetraploid induction rate achieved when using mutagens, (2) to evaluate the agronomic traits of *C. willmottianum* in the field and achieve tetraploid strains with greater compactness and larger flowers, and (3) to assess the photosynthetic capacity to test the development of tetraploid strains with higher CO₂ assimilation efficiency. Our results can fill a gap by creating a protocol for polyploidization of the genus *Cerastigma* based on tissue cultures and chemical mutagenesis. Moreover, they can also be applied to enrich the varieties of multifunctional plants in cities and provide a scientific basis for selecting plants that capture CO₂ efficaciously to achieve carbon neutrality.

Materials and methods

Plant materials

Sterile apical shoots with buds and leaves were collected from our previously established aseptic seedlings of *C. willmottianum* ($2n = 2x = 12$). The experiment was conducted in the laboratory of the Landscape Architecture Institute at Sichuan Agricultural University, Chengdu, China.

The mutagens included in this study were colchicine (Shanghai Meryer Chemical Technology Co., Ltd., China), trifluralin (Zhenjiang Jiansu Pesticide Chemical Co., Ltd., China) and pendimethalin (India United Phosphates Co., Ltd.).

Polyploid induction based on tissue cultures and chemical mutagenesis

Filter-sterilized 2% DMSO was first added to the prepared medium (4.42 g L⁻¹ MS + 30 g L⁻¹ sucrose + 7 g L⁻¹ agar; pH = 5.80; high-pressure sterilization), and filter-sterilized colchicine, trifluralin and pendimethalin were then added. For the sake of clarity, medium supplemented with the mutagens is referred to as mutagenic medium. Then, cut apical buds of aseptic seedlings were inoculated on solid mutagenic medium on an ultraclean bench. The concentrations and durations of the three mutagen treatments were determined by a secondary saturated D-optimal design (Table S1). There was a total of 18 treatments with three

mutagens and 2 controls, which were referred to as Control 1 (2% DMSO) and Control 2 (the blank control). Each treatment contained 30 explants, and the experiment was repeated 3 times. After induction, the explants were rinsed in sterile water 4–5 times; then, the portion of the stem that was in contact with the mutagenic medium was excised, and the rest of the plant material was transferred to and continuously incubated in solid rooting medium (2.21 g L⁻¹ MS + 30 g L⁻¹ sucrose + 7 g L⁻¹ agar + 0.5 mg L⁻¹ IBA; pH 5.80; high-pressure sterilization) (referring to methods described by Hu et al. 2019) for 45 days. During this period, the survival rate and morphological variation rate were observed and recorded. In particular, to obtain purified plantlets, we cut plant used the method of serial generation by cutting plant organs. That is, the apical buds of explants with observed morphological variations were cut and subcultured in solid rooting medium every 15–20 days for 5–8 generations. The plants were held in the culture media at room temperature (25 ± 1 °C), with humidity maintained at $70 \pm 5\%$, a photoperiod of 14 h/day, and a light intensity of 1500–2000 lx.

Tetraploid identification

Stomatal measurement

Preliminarily, plantlets with stable morphological variations were screened from the purifications, and stomata were observed and measured under a microscope (Olympus BX53 + DP80, Tokyo, Japan) with a 40× objective lens (referring to methods described by Zhang and Gao (2021) with slight modifications). In particular, three leaves from each plantlet were measured, and each leaf was measured 3 times, three randomly sampled microscopic-field areas of each production were measured, for a total of 27 samples. In comparison to diploid plants, there were plantlets with morphological variations (named potential plantlets and abbreviated PP); these plantlets, which had enlarged and relatively low numbers of stomata, were regarded as having stomatal variation and were selected for the next test.

Flow cytometry analysis and chromosome counting

The nuclear DNA content of the PP, compared with that of diploid plants, i.e., the internal reference, was estimated using FCM (BD Accuri® C6 Plus, USA). PPs having a doubled nuclear DNA content were selected to conduct chromosome counting. The experiments were conducted following the methods described by Jiang et al. (2019) with slight modifications.

Anatomical characteristics and ornamental traits of the tetraploids

To compare diploids with tetraploids, we first analyzed their anatomical characteristics. The anatomical structures of roots, stems and leaves of plants with the two ploidy levels (10 of each) were compared by examination under an Olympus BX53 + DP80 microscope. Then, we measured the stomata of plants with the two ploidy levels (30 of each); the method used for this analysis was the same as that applied for stomatal measurement (referring to methods described by Zhang and Gao (2021) with slight modifications). To clarify, the ‘two ploidy levels’ mentioned in this paragraph refer to uniformly grown aseptically grown diploids and tetraploids (90 days old).

To quantify ornamental characteristics, first, traits such as plant height of potted diploids and tetraploids (60 of each) were measured (referring to methods described by Zhang et al. (2020b) with slight modifications). Then, the values of the LAI (leaf area index, equal to total leaf area/the ground shadow of the plant canopy, abbreviated S1/S2) for plants with the two ploidy levels (30 of each) was determined following the methods described by ASEF et al. (2020) and Silva et al. (2019) with slight modifications. To clarify, the ‘two ploidy levels’ mentioned in this paragraph refer to uniformly grown potted diploids and tetraploids (180 days old).

CO₂ fixation ability of tetraploids

According to the morphological data, we randomly chose ten tetraploids with greater compactness and larger flowers and ten diploids; these plants were potted during the same growth period and were used for the following experiments.

Diurnal gas exchange patterns under field conditions

Parameters such as the net photosynthetic rate (P_n), intercellular carbon dioxide concentration (C_i), stomatal conductance (G_s), transpiration (Tr), water use efficiency (WUE) and vapor pressure deficit (VPD) were measured by a CIRAS-3 portable photosynthesis system (American PP Systems Co., Ltd.) (referring to methods described by García-García et al. (2020) with slight modifications).

Chlorophyll fluorimeter measurements

The minimal values of chlorophyll a fluorescence (F_o), maximum fluorescence (F_m) and variable fluorescence of PS II (F_v , equal to $(F_m - F_o)/F_m$) were measured by a Handy PEA fluorimeter (Hansatech, UK). In the present study, the third to fifth leaves of each plantlet from top to bottom were measured from 9:00 to 11:00 on a sunny morning (referring to the protocols described by Jiang et al. (2019) and García-García et al. (2020), with slight modifications).

Photosynthetic pigments

The chlorophyll a (Chl a), chlorophyll b (Chl b), total chlorophyll (Chl a + b) and chlorophyll a/chlorophyll b (Chl a/b) contents were measured at 646 nm and 663 nm by a full wavelength Multiskan GO microplate reader (Thermo Fisher Science Oy, Ratatie 2, FI-01620 Vantaa, Finland) (referring to methods described by Warner and Edwards (1989), with slight modifications).

Statistical analysis

In this study, the data were analyzed by IBM SPSS statistics 22.0. The data were assessed by ANOVA with the

Fig. 1 FCM identification of diploids (a) and tetraploids (b)

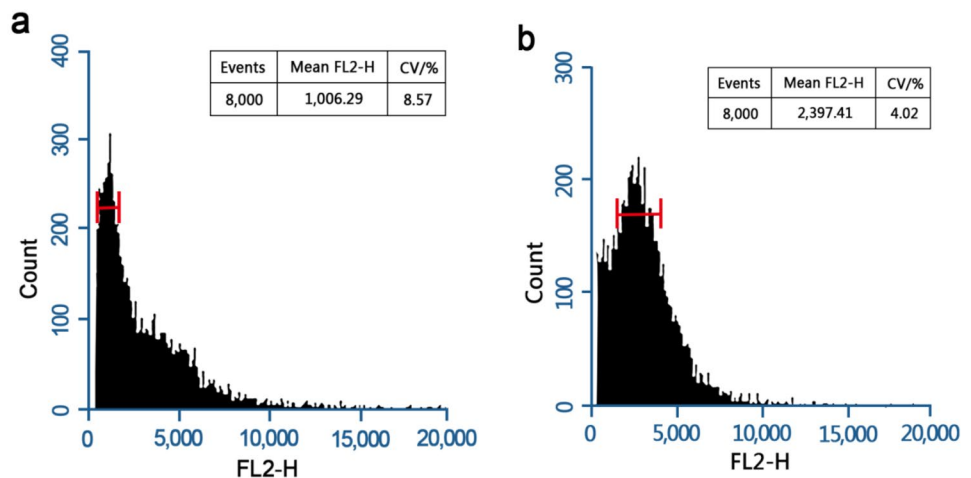


Fig. 2 Comparison of chromosome number in the root tip between diploids and tetraploids. **a** Diploid root tip cells ($2n=2x=12$); **b** Tetraploid root tip cells ($2n=4x=24$)

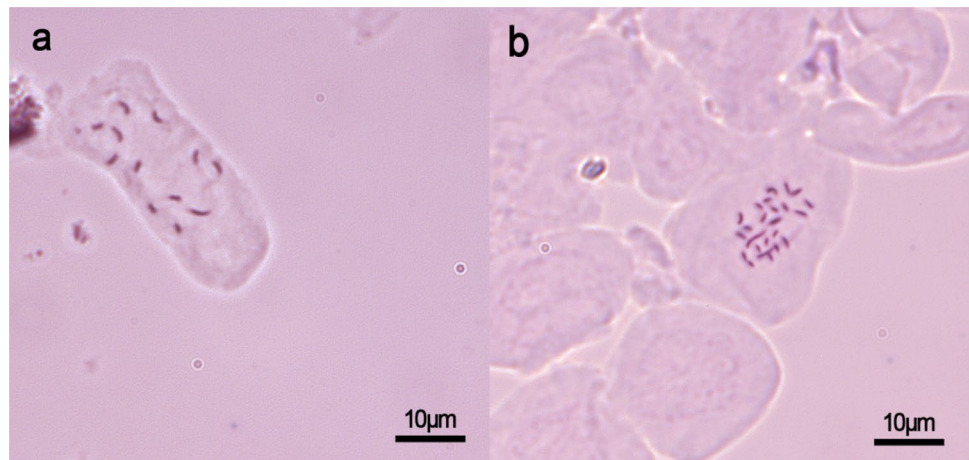


Table 1 Effect of DMSO and three mutagens on a survival rate of apical buds

Treatment	DMSO concentration (%)	Number of samples ^a	Survival rate ^b (%)
Colchicine	2.00	540	13.16 ± 0.36 ^d
Pendimethalin	2.00	540	53.38 ± 2.52 ^{bc}
Trifluralin	2.00	540	46.17 ± 5.22 ^c
Control 1	2.00	540	64.50 ± 1.12 ^{ab}
Control 2	0.00	540	67.55 ± 6.70 ^a

Different lowercase letters based on Duncan's test at $P \leq 0.05$ indicating significant differences among treatments by one-way ANOVA

Number of samples^a = total of three replicates

Survival rate^b (%) = (number of survival samples/number of treated samples) * 100%

^bData representing the mean ± SE of three replicates

HSD-Tukey test or Pearson correlation analysis. A P value less than 0.05 was considered a significant difference or significant correlation. The tables and graphs were made by Microsoft Excel 2010 and Adobe Photoshop CC 2018.

Results

Tetraploid induction by trifluralin, pendimethalin and colchicine

In this study, a total of 109 PPs were screened from 469 surviving aseptic seedlings by a combination of morphological observation and stomatal measurement. Then, we conducted an analysis with FCM. As shown in Fig. 1, the fluorescence intensity of the main peak of the tetraploids was approximately twice as strong as that of the diploids. Next, the chromosome numbers were determined (Fig. 2).

During a period of normal culture for 45 days after induction, there were differences in the survival rate between the two controls and treatments with three mutagens (Table 1). The analysis performed with SPSS showed that there was no significant difference in the survival rate between Control 1 (supplemented with 2% DMSO) and Control 2 (the blank control) ($P = 0.601$). Furthermore, there was a significant difference in the survival rate between Control 1 and the colchicine-treated group ($P = 0.000$). Combined with observations, these results indicated that colchicine was extremely harmful to the explants and that they experienced difficulty rooting, leading to curly leaves and brown leaf margins after treatment. The average survival rate of all the explants treated with colchicine was as low as 13.16%. Furthermore, colchicine failed to induce tetraploidy (Table 2). As shown in Table 2, the polyploidization rate of trifluralin was higher than that of pendimethalin. Further multifactor variance analysis showed that treatment with trifluralin, the treatment concentration, the treatment duration and their interaction all led to significant differences ($P = 0.000$, $P = 0.001$ and $P = 0.003$, respectively). The effect of the concentration was greater than that of the duration (partial Eta squared values: concentration (0.740) > duration (0.691)). Taken together, the results of this study indicated that an optimal protocol for tetraploid induction of *C. willmottianum* was treating aseptic apical buds with 1200.0 μM trifluralin and 2% DMSO for 7 days, with a survival rate of 74.45% and a polyploidization rate of 17.78%.

Anatomical and morphological features of the tetraploids

To compare the diploids with tetraploids, we first observed and analyzed their anatomical structures (Tables 3, 4; Fig. 3a-j). For the tetraploid plants, except for the stomatal

Table 2 Effect of three mutagens on tetraploid induction of *Cerastigma Willmottianum* Stapf

Treatment	Concentration (%/μM)	Exposure duration (day)	Number of samples	Survival rate (%)	Morphological variation rate ¹ (%)	Stomatal variation rate ² (%)	Tetraploid induction rate ³ (%)
1	0.05	7.0	90	51.11±7.78 ^{abcd}	4.44±2.94 ^{cd}	2.22±1.11 ^f	0.00±0.00 ^f
	400.0	7.0	90	62.78±10.55 ^{abc}	9.72±5.01 ^{bcd}	6.95±1.69 ^{cdef}	4.72±1.00 ^{ef}
	400.0	7.0	90	57.78±6.76 ^{abcd}	10.00±8.82 ^{bcd}	6.67±1.93 ^{def}	5.56±1.11 ^{ef}
2	0.20	7.0	90	8.89±5.88 ^{fg}	1.11±1.11 ^d	0.00±0.00 ^f	0.00±0.00 ^f
	1200.0	7.0	90	69.17±9.46 ^{ab}	11.94±4.82 ^{abcd}	9.72±1.69 ^{bcd}	8.61±1.94 ^{cde}
	1200.0	7.0	90	74.45±13.10 ^a	18.89±12.62 ^{ab}	18.89±2.22 ^a	17.78±1.11 ^a
3	0.05	21.0	90	17.04±6.10 ^{efg}	0.00±0.00 ^d	0.00±0.00 ^f	0.00±0.00 ^f
	400.0	21.0	90	45.00±11.67 ^{bcd}	15.56±1.11 ^{abc}	14.45±2.22 ^{abc}	13.34±3.33 ^{abc}
	400.0	21.0	90	39.63±10.76 ^{cde}	24.44±8.39 ^a	14.82±1.85 ^{ab}	12.59±2.06 ^{abc}
4	0.11	12.2	90	4.63±4.24 ^g	0.00±0.00 ^d	0.00±0.00 ^f	0.00±0.00 ^f
	694.8	12.2	90	58.15±7.84 ^{abcd}	24.44±5.88 ^a	13.70±1.62 ^{abcd}	11.48±0.98 ^{bcd}
	694.8	12.2	90	31.85±5.19 ^{def}	14.81±4.63 ^{abc}	12.22±2.22 ^{abcde}	10.37±0.37 ^{bcd}
5	0.20	19.5	90	0.00±0.00 ^g	0.00±0.00 ^d	0.00±0.00 ^f	0.00±0.00 ^f
	1200.0	19.5	90	42.96±10.37 ^{bcd}	18.52±6.55 ^{ab}	18.15±4.82 ^a	15.18±3.65 ^{ab}
	1200.0	19.5	90	37.78±8.68 ^{cde}	15.56±6.97 ^{abc}	10.00±5.09 ^{bcd}	7.78±2.94 ^{cde}
6	0.17	21.0	90	0.00±0.00 ^g	0.00±0.00 ^d	0.00±0.00 ^f	0.00±0.00 ^f
	1115.6	21.0	90	42.22±7.78 ^{bcd}	14.63±5.65 ^{abc}	5.93±3.23 ^{ef}	5.93±3.23 ^{def}
	1115.6	21.0	90	35.56±8.01 ^{cde}	15.56±1.93 ^{abc}	10.00±1.92 ^{bcd}	7.78±1.11 ^{cde}

Different lowercase letters based on Duncan's test at $P \leq 0.05$ indicating significant differences among treatments by one-way ANOVA; The data marked in three colors ' ■ ■ ■ ', respectively representing induction using colchicine, pendimethalin and trifluralin

Morphological variation rate¹ (%)=(number of morphology-variation samples/number of treated samples)*100%

Stomatal variation rate² (%)=(number of stomata-variation samples/number of treated samples)*100%

Tetraploid induction rate³ (%)=(number of tetraploids/number of treated samples)*100%

^{1, 2, 3}Data representing the mean ± SE (standard error) of three replicates

density which decreased, increases were seen in all of the structures, including the diameters of the root and stem; thicknesses of the leaf, leaf epidermal cells, palisade tissue, spongy tissue, and main vein; and the lengths and widths of the stomata and guard cells. Apart from the thickness of the leaf epidermal cells ($P = 0.112$), the other parameters were all significantly different (spongy tissue: $P = 0.002$; remaining structures: $P = 0.000$).

Among the morphological characteristics of the tetraploids (Table 5; Fig. 4a-f), plant height, stem diameter, canopy diameter, leaf length, leaf shape index and internode length were reduced; however, leaf width, leaf thickness, flower diameter, cylinder length and leaf area index increased. Except for the stem diameter, leaf thickness, internode length and leaf area index ($P = 0.343$, $P = 0.490$, $P = 0.061$ and $P = 0.384$, respectively), significant

Table 3 Comparison of tissue structure between diploids and tetraploids

Plant	Root diameter (μm)	Stem diameter (μm)	Leaf thickness (μm)	Leaf epidermal cell thickness (μm)	Leaf palisade tissue thickness (μm)	Leaf spongy tissue thickness (μm)	Leaf main vein thickness (μm)
2x	679.41 ± 39.89 ^b	1013.25 ± 69.02 ^b	295.81 ± 2.37 ^b	13.02 ± 0.35	34.66 ± 1.16 ^b	48.16 ± 3.39 ^b	295.81 ± 2.37 ^b
4x	1024.00 ± 131.63 ^a	1525.37 ± 95.53 ^a	455.011 ± 1.58 ^a	14.43 ± 0.67	60.06 ± 2.78 ^a	69.04 ± 1.17 ^a	455.01 ± 1.58 ^a
Difference ^a (%)	50.72	50.54	53.82	10.83	73.28	43.36	53.82

Different lowercase letters based on Duncan's test at $P \leq 0.05$ indicating significant differences between diploids and tetraploids by two-sample t-test

Difference^a (%) = (indicator of 4x/the same indicator as 4x of 2x - 1) * 100%. the calculation method of referred 'difference' later in the table being the same as it

Table 4 Comparison of stomatal characteristics of diploids and tetraploids

Ploidy	Stomata		Guard cells		Stomatal density (mm ²)
	Length (μm)	Width (μm)	Length (μm)	Width (μm)	
2x	23.35 ± 2.19 ^b	13.95 ± 2.62	38.77 ± 3.15 ^b	33.13 ± 4.23	131.88 ± 21.65 ^a
4x	29.49 ± 3.16 ^a	14.60 ± 4.36	46.14 ± 4.00 ^a	35.80 ± 5.91	63.54 ± 14.10 ^b
Difference (%)	26.30	4.66	19.01	8.06	-48.18

Different lowercase letters based on Duncan's test at $P \leq 0.05$ indicating significant differences between diploids and tetraploids by two-sample t-test

differences were observed for all the parameters (all $P = 0.000$). In addition, we found that white granules (Fig. 4g-j) on the leaf surface and epidermal hairs (Fig. 4k, l) of the leaf margin were more obvious for tetraploids than for diploids.

CO₂ fixation ability of tetraploids

As shown in Fig. 5, the contents of Chl a, Chl b and total chlorophyll were higher in the tetraploids than in the diploids, and there were significant differences in the contents between ploidy levels (all $P = 0.000$). Chl a/b was slightly lower in the tetraploids than in the diploids but showed no significant difference ($P = 0.079$). Furthermore, F_m/F_o , F_v/F_o and F_v/F_m , which were determined by chlorophyll fluorimeter measurement, were not significantly different between the diploid and tetraploid plants ($P = 1.000$, $P = 1.000$ and $P = 0.742$, respectively) (Table 5). For the parameters of the diurnal gas exchange patterns between the two ploidy levels (Fig. 6), first, the same trend (a bimodal curve) was observed for Pn, Gs and Ci, which increased to a maximum value and then decreased gradually. The Ci values of the diploids and tetraploids both peaked at 9:30 ($420.44 \pm 14.06 \mu\text{mol}\cdot\text{mol}^{-1}$ and $377.44 \pm 13.09 \mu\text{mol}\cdot\text{mol}^{-1}$, respectively). However, throughout the day, the maximum Pn and Gs values of the tetraploid plants ($8.24 \pm 1.19 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and $179.78 \pm 5.99 \text{mmol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, respectively) were reached 1 h later than those of

the diploid plants ($6.37 \pm 0.35 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and $229.556 \pm 57.82 \text{mmol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, respectively). Except from 13:30 to 15:30, the value of Ci in the tetraploids was always lower than that in the diploids. From the correlation coefficients of the daily pattern of photosynthetic characteristic parameters (Table 6), we found that there was an extremely significant positive correlation of the Pn and Gs between the diploids and tetraploids.

Discussion

An optimal protocol for tissue culture and chemical mutagenesis for the induction and identification of tetraploids

Artificial polyploidization with chemical mutagens is the most effective and important way to acquire polyploids of garden plants, and it has many advantages, such as a low cost, simplicity, strong specificity and ability to produce a wide spectrum of mutations (Eng and Ho 2019). Currently, colchicine is widely used as an antimetabolic agent. However, this chemical is more toxic than alternatives, and its ability to depolymerize plant tubulins is not as good as that of dinitroaniline herbicides, including pendimethalin and trifluralin (Castillo et al. 2009; Eng and Ho 2019). In this study, the colchicine treatment produced explants with curly leaves, brown leaf margins and very low rooting rates,

Fig. 3 Comparison of anatomical structures of diploids (left) and tetraploids (right). Root cross-section (**a, b**); Stem cross-section (**c, d**); Leaf cross-section (**e, f, g, h**. SA, stomata; UE, upper epidermal; LE, lower epidermal; MV, main vein; PT, palisade tissue; ST, spongy tissue); Guard cells and stomata (**i, j**)

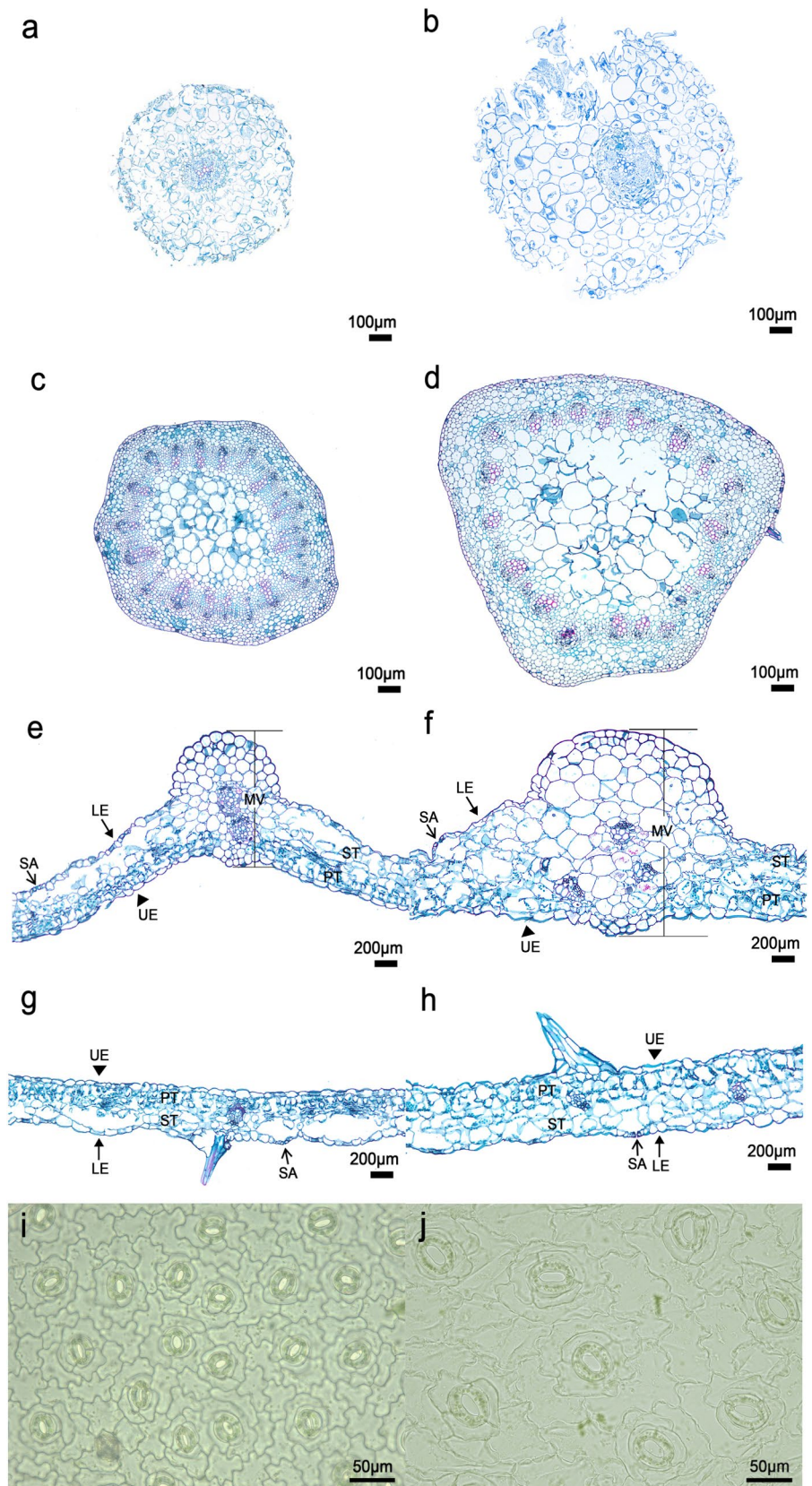


Table 5 Comparison of morphological characteristics and fluorescence parameters of diploids and tetraploids

Indicators	Diploids	Tetraploids	Difference(%)
Plant height (cm)	17.50±0.74 ^a	7.57±0.58 ^b	-56.74
Stem diameter (mm)	2.90±0.12	2.75±0.10	-5.17
Canopy diameter (cm)	11.83±0.42 ^a	5.56±0.32 ^b	-53.00
Leaf length (mm)	26.41±0.47 ^a	21.26±0.70 ^b	-19.50
Petiole length (mm)	9.43±0.22 ^a	7.08±0.23 ^b	-24.92
Leaf width (mm)	18.10±0.34 ^b	21.04±0.63 ^a	16.24
Leaf thickness (mm)	0.44±0.15	0.54±0.01	22.73
Leaf shape index	1.47±0.02 ^a	1.01±0.01 ^b	-31.29
Internode length (mm)	7.78±0.27	6.60±0.56	-15.17
Flower diameter(mm)	20.06±0.20 ^b	23.65±0.11 ^a	17.90
Cylinder length (mm)	22.22±0.20 ^b	25.76±0.14 ^a	15.93
Leaf area index	2.27±0.12	3.27±0.91	44.05
Fo (μmol m ⁻² s ⁻¹)	531.67±15.99	543.56±23.52	2.24
Fm/Fo	6.48±0.21	6.48±0.27	0.00
Fv/Fo	5.48±0.27	5.48±0.21	0.00
Fv/Fm	0.845±0.009	0.845±0.003	0.00

Different lowercase letters based on Duncan's test at $P \leq 0.05$ indicating significant differences between diploids and tetraploids by two-sample t-test

with an average survival rate that was lower than those of pendimethalin and trifluralin. Therefore, in terms of explant damage, the colchicine treatment resulted in the most damage, followed by trifluralin and pendimethalin; this result is consistent with those of previous studies (Aslam et al. 2017; Ren et al. 2018; Jiang et al. 2019). Tetraploids were induced successfully by colchicine in maize (*Zea mays* L.) (Aslam et al. 2017) and tillered onion (*Allium cepa* L. var. *aggregatum* G. Don) (Ren et al. 2018), but the application of this chemical failed to produce tetraploid plants in this study. We speculate that plants of different genotypes and explant types have different sensitivities and tolerances to different mutagens (Eng and Ho 2019; Niazi and Nalousi 2020). Our research suggests that aseptic apical buds may not be suitable for colchicine treatment, that the treatment time at low concentrations was too short to double the number of chromosomes in *C. willmottianum*, or that colchicine was so harmful to the explants exposed to higher concentration for a long time that many dead explants may be polyploids (Eng and Ho 2019).

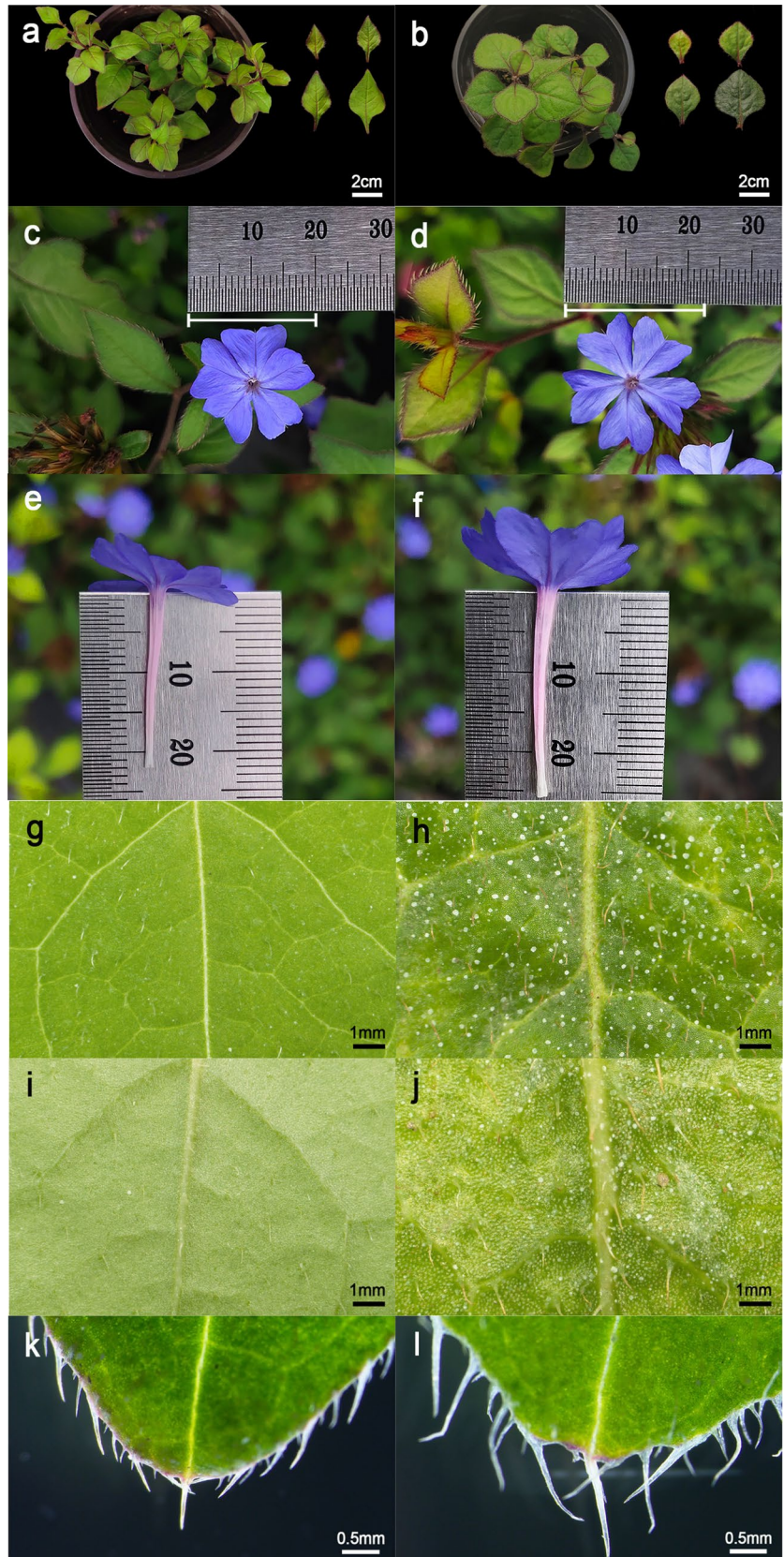
DMSO is a sulfur-containing organic compound used as a penetrating agent. It has been reported that adding 1%-2% DMSO to treatments can promote the absorption of mutagenic solutions by plant cells, causing a higher

polyploidization rate. However, the toxicity of DMSO may affect the survival rate of plants (Eng and Ho 2019). Hamill et al. (1992) found that treatment with 2% DMSO increased the mortality rate of banana (*Musa nana* Lour., 'SH-3362'). In our study, Control 1 was treated with 2% DMSO, and the survival rate of plants in Control 1 was nearly equal to that in Control 2, which did not contain DMSO. DMSO had few side effects on the survival rate in this study, which differs from previously reported results.

The identification of polyploids is vital to determining polyploid induction efficiency. To date, polyploid identification methods have included morphological observation, stomatal measurement, FCM and chromosome counting (Eng and Ho 2019). In general, for the tetraploids, the lengths and widths of the stomata were greater than those of the diploids, while the stomatal density was lower in the tetraploids; similar findings were observed for Chinese privet (*Ligustrum sinense* Lour.) (Fetouh et al. 2020) and cultivated gerbera (*Gerbera hybrida*; Asteraceae) (Bhattarai et al. 2021). Stomatal measurements can be used as a preliminary screening method to determine whether plantlets are polyploids. However, stomatal mutations and environmental factors can also cause changes in stomata. In short, additional analyses are needed to confirm the presence of polyploidy (Eng and Ho 2019). FCM is a fast and effective method to estimate the nuclear DNA content of samples and determine the ploidy level. However, the results will be affected by the freshness of the samples and the cytoplasmic compounds. Thus, FCM is used only as an indirect method for screening large numbers of explants after induction with its time-saving and increased efficiency of identification (Ochatt et al. 2011; Talebi et al. 2017; Eng and Ho 2019; Zhang and Gao 2020). Although chromosome counting is direct and intuitive, it is necessary to conduct chromosome preparation. Therefore, using this method to identify the polyploidy level is difficult on a large scale (Ochatt et al. 2011).

To date, four methods have been applied for the identification of ploidy level (Madani et al. 2015; Ravandi et al. 2013; Hannweg et al. 2016; Talebi et al. 2017; Fetouh et al. 2020; Zhang and Gao 2020, 2021; Zhang et al. 2020b); however, these methods are relatively time-consuming and laborious, and there are no obvious advantages among them. Ren et al. (2018) combined the four methods in the following order to identify polyploids: morphological observation, stomatal size measurement, chromosome counting, and FCM. However, this process requires a certain amount of time, and fresh roots are required to count the number of chromosomes. Chromosome production requires highly skilled experimentalists and easily fails. Large-scale chromosome identification is time-consuming. FCM can be performed with fresh materials obtained at any time and is carried out in a stepwise manner under an experimental protocol with demonstrated success; furthermore, application of

Fig. 4 Comparison of morphological characteristics of diploids (left) and tetraploids (right). Plantlets and leaves (**a**, **b**); Flowers (**c**, **d**); Cylinders (**e**, **f**); White granules of adaxial (**g**, **h**) and abaxial leaf (**i**, **j**); Leaf epidermal hairs at leaf margin (**k**, **l**)



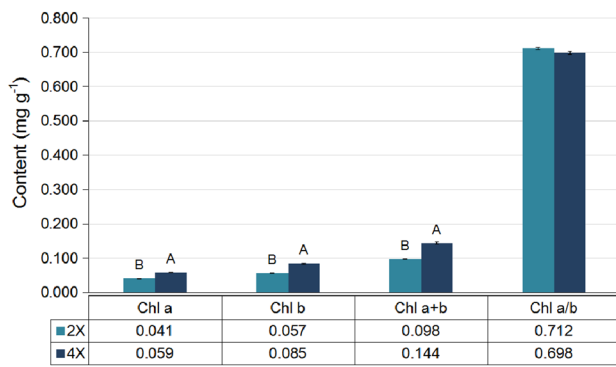


Fig. 5 Comparison of photosynthetic pigment contents of diploids and tetraploids (Chl a, Chl b, Chl a+b, Chl a/b). Value representing the mean \pm SE (standard error) of ten samples of each ploidy. Different capital letters based on Duncan's test at $P \leq 0.05$ indicating significant differences between diploids and tetraploids by two-sample t-test

this method can save time (Eng and Ho 2019). Therefore, we believe that it is more efficient and practical to conduct FCM analysis before chromosome counting for polyploidy identification. In this study, we developed an efficiently integrated method for screening and identifying polyploids that includes morphological observation, stomatal measurement, DNA content analysis, and chromosome counting.

Assessment of ornamental features and CO₂ fixation ability of tetraploids

Generally, polyploidization increases the size of cells, causing changes in their anatomical structure and morphological characteristics. It also affects the photosynthetic process (Vyas et al. 2007; Coate et al. 2012; Eng and Ho 2019). In this study, we observed and analyzed the anatomical structures of plants at two ploidy levels. We found that the sizes of the cells and tissues in the roots, stems and leaves of the tetraploids increased in comparison to those of the diploids, which is consistent with the findings of Jiang et al. (2019). Morphologically, polyploidization causes a 'giga' effect,

resulting in larger organs (leaves and flowers) and changes in leaf shape (Hannweg et al. 2016; Mariana et al. 2016; Niazi and Naloussi 2020). This study found that tetraploids had shortened internodes, dwarf stature and canopy diameters that were reduced by half. The *C. willmottianum* tetraploids were transformed into more compact plants than the diploids. Furthermore, there was a significant difference in the leaf shape index, and the leaf shape changed from an obovate rhombus to a heart shape. These results are similar to the results of Ravandi et al. (2013), Fetouh et al. (2016), Hannweg et al. (2016), and Mo et al. (2020).

It has been reported that CO₂ fixation in leaves is proportional to the LAI (Doughty and Goulden 2008; Choi et al. 2014). Field measurements of the LAI of the tetraploids were higher than those of the diploids, which provides preliminary evidence that the tetraploids absorbed more CO₂ for fixation. Photosystem II (PS II) and photosystem I (PS I), included in the photoreaction stage, represent the ability of plants to fix CO₂ and synthesize carbohydrates. Furthermore, plants use chlorophyll to collect and transmit light energy to the reaction center for the photoreaction stage of photosynthesis, synthesizing NADPH and ATP, which are used to fix CO₂ in the carbon reaction stage. Thus, the chlorophyll content can, to some extent, represent and is generally positively correlated with the photosynthetic capacity of a plant (Jajoo et al. 2001; Cenzano et al. 2013; Chen et al. 2019; Eng and Ho 2019). In this study, the contents of total chlorophyll, Chl a and Chl b in the tetraploids were all significantly higher than those in the diploids. This demonstrated that the tetraploids had a higher ability to collect and transmit light energy to PS II and PS I, which is consistent with the results of Madani et al. (2015) and Cao et al. (2018). *F_o*, *F_m* and *F_v* in PS II are measured by a chlorophyll fluorimeter to analyze their state of reactivity and reflect the physiological conditions of photosynthesis (Demmig-Adams et al. 1989; Cai and Feng 2020). This study found that *F_m/F_o*, *F_v/F_o* and *F_v/F_m* were almost equal between the diploid and tetraploid plants. In other words, the transportation of electrons of PS II, the activity of PS II and the maximal quantum yield of PS II were almost equal to each other. The *P_n*, determined

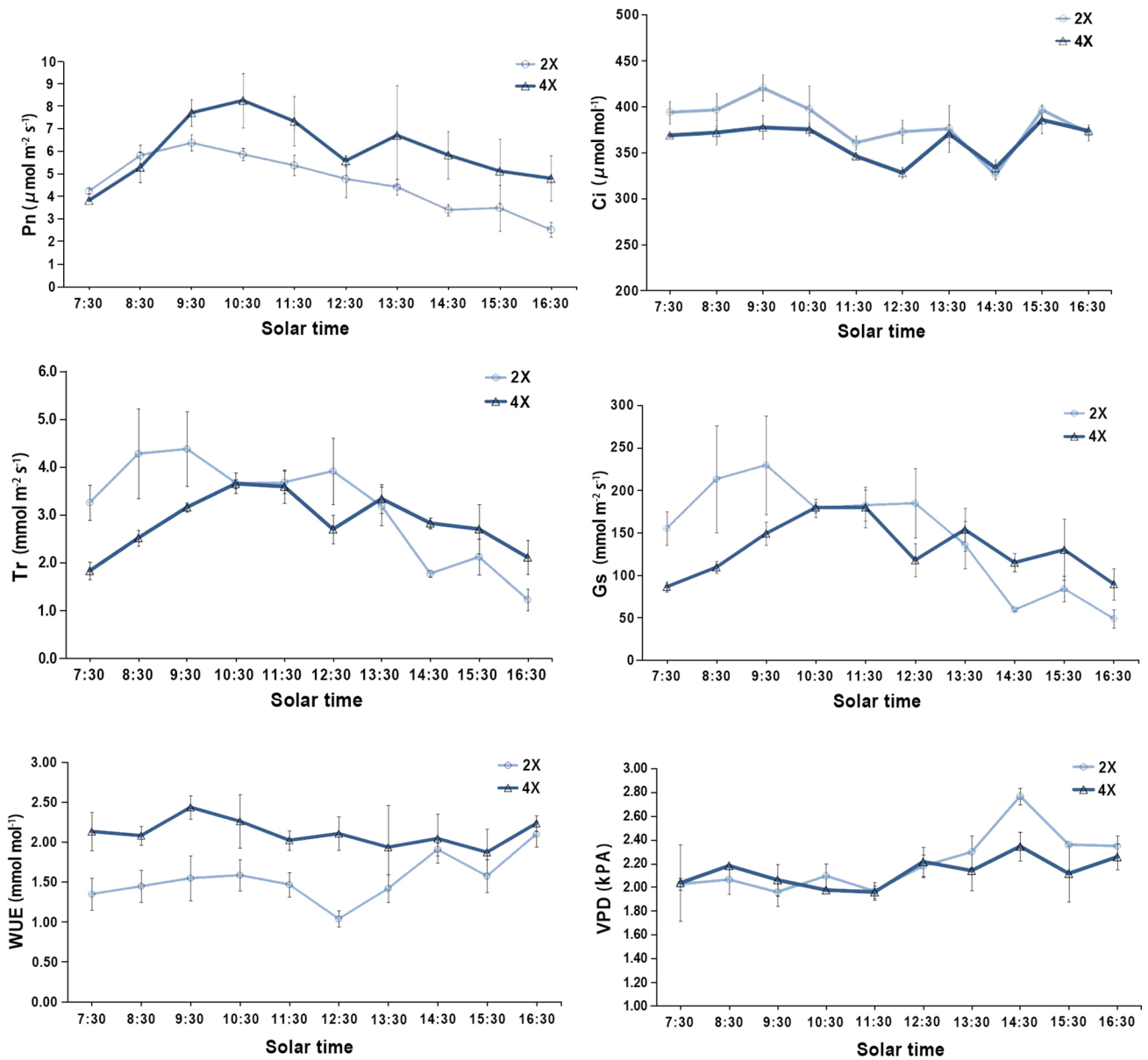


Fig. 6 Comparison of the parameters (Pn, Ci, Tr, Gs, WUE, VPD) of the diurnal gas exchange patterns between diploids and tetraploids. Value representing the mean \pm SE (standard error) of ten samples of each ploidy

Table 6 Correlation coefficients of the parameters of the diurnal gas exchange patterns between diploids and tetraploids

Ploidy	Pn	Ci	Tr	Gs	WUE
Diploids					
Ci	0.536				
Tr	0.944**	0.539			
Gs	0.952**	0.585	0.922**		
WUE	-0.529	-0.317	-0.748*	-0.688*	
VPD	-0.732*	-0.717*	-0.785**	-0.836**	0.541
Tetra-ploids					
Ci	0.014				
Tr	0.943**	-0.081			
Gs	0.919**	0.040	0.973**		
WUE	0.333	0.155	0.003	-0.014	
VPD	-0.461	-0.380	-0.441	-0.628	-0.167

**Correlation extremely significant level ($P \leq 0.01$), *Correlation significant level ($P \leq 0.05$). A value of plus and minus indicating a positive and negative correlation respectively

by both PS II and PS I, is proportional to the CO₂ assimilation ability of a plant (Jajoo et al. 2001). This study found that the Pn of the tetraploids was always higher than that of the diploids, which meant that there was more CO₂ fixation in the tetraploids. Therefore, we speculate that the PS I of the tetraploids had stronger reactivity and thus could

synthesize more NADPH and ATP to be used in the carbon reaction stage or that the tetraploids had a greater ability to fix CO₂ in the carbon reaction stage. Moreover, some studies have shown that when Pn and Gs are positively correlated, a higher Gs causes a higher Ci with more CO₂ available for assimilation (Yuan et al. 2018; Chen et al. 2019, 2021). In this study, we found that there was an extremely significant positive correlation of the Pn and Gs between the diploids and tetraploids. The Gs and Ci of the tetraploids on most of the days were both lower than those of the diploids, according to the measurements of the diurnal gas exchange patterns. In other words, the stomatal conductance and intercellular CO₂ concentrations of the tetraploids were both lower than those of the diploids. However, the Pn of the tetraploids was higher than that of the diploids on most of the days. Further analysis indicated that tetraploids have a higher efficiency of CO₂ fixation. In conclusion, for the tetraploids, the related results of greater LAI, higher chlorophyll content,

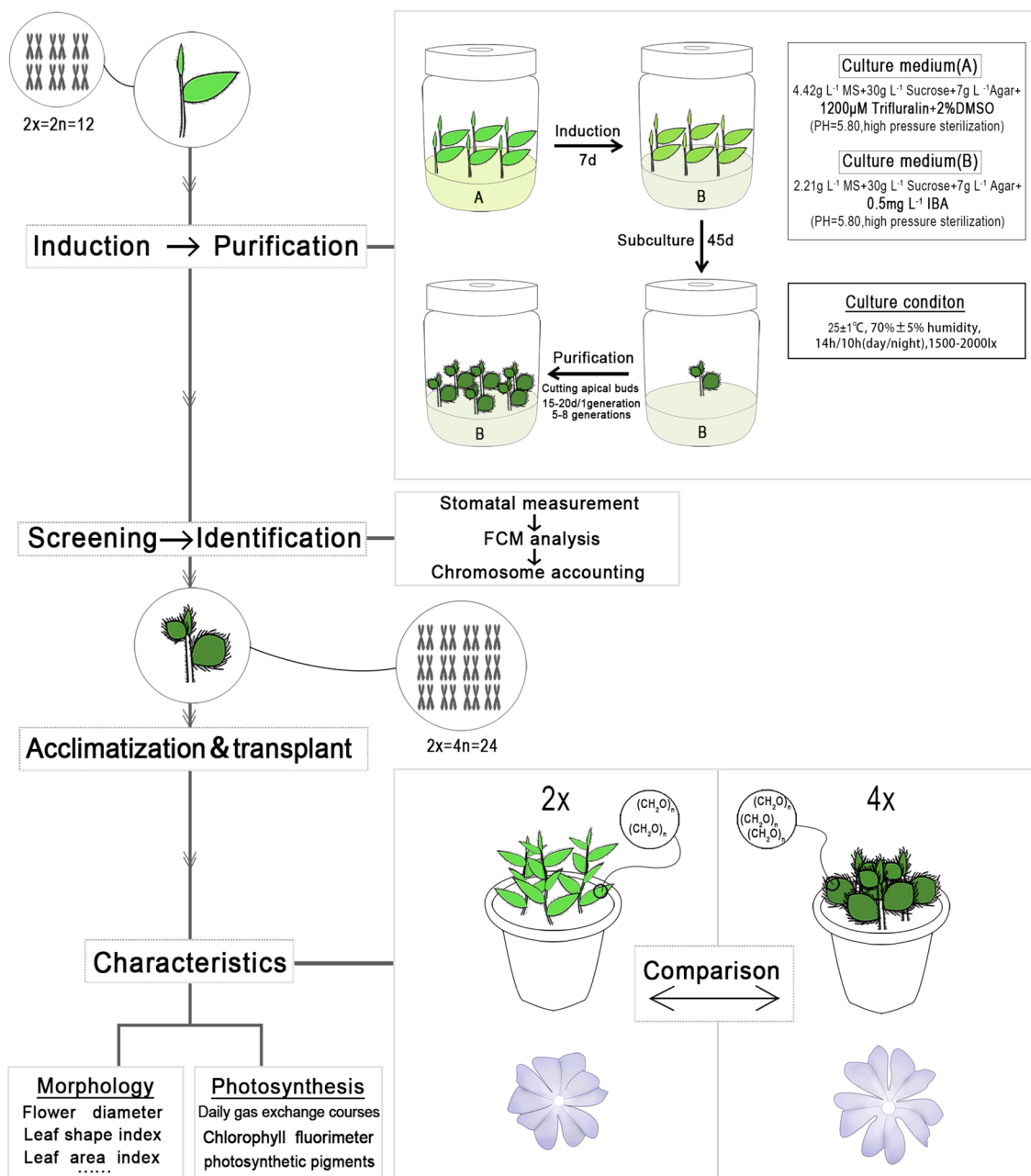


Fig. 7 an efficiently integrated protocol for polyploidization of *C. Willmottianum* and tetraploid characteristics

and higher Pn with a positive correlation with Gs verified that the efficiency of CO₂ assimilation by the tetraploids was higher than that by the diploids.

Conclusions

In this study, we have unprecedentedly developed an efficiently integrated protocol for the polyploidization, purification, screening and identification of *C. willmottianum*

(Fig. 7). Compared with diploids, tetraploids have unique characteristics, including their morphology, anatomy and photosynthetic ability. Tetraploid plants have larger flowers, heart-shaped leaves and greater compactness than diploid plants, greatly increasing their ornamental value. They have a greater ability to fix CO₂, with a higher efficiency of CO₂ assimilation, during the photosynthetic process. Taken together, these results indicate that the novel tetraploid germplasm can not only enrich the supply of novel plants in green areas and landscapes but also absorb excess CO₂

from the atmosphere through the photosynthetic process to achieve carbon neutrality.

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Author contributions LSS, SPG and TL designed the research. SPG, TL and LJY supervised the whole research. LSS performed most of the experiments, analyzed the data and wrote the manuscript. YFD, QL conducted part of the experiments. JNL and ZAZ proposed helpful suggestions and collected resources. Moreover, thanks to people such as YML, WJL, LP, SCJ helped in the research.

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Declarations

Conflict of interest The authors declare no conflict of interest.

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